# CED-4—The Third Horseman of Apoptosis

# Minireview

**David L. Vaux**
The Walter and Eliza Hall
  Institute of Medical Research
Post Office Royal Melbourne Hospital 3050
Victoria
Australia

The remarkable conservation of physiological cell death mechanisms from nematodes to humans has allowed the genetic pathways of programmed cell death determined in Caenorhabditis elegans to act as a framework for understanding the biology of apoptosis in mammalian cells. However, it has been unclear whether the C. elegans cell death gene *ced-4* would have a parallel in mammalian cell death. In this issue of *Cell*, Zou et al. (1997) report the biochemical identification of a human cell death protein whose sequence resembles the nematode protein CED-4, providing a spectacular demonstration of the combined power of genetic analysis of a simple organism with biochemistry of mammalian cells.

### The Genetic Framework

Although the first cell death gene to be characterized at the molecular level was a mammalian gene, *bcl-2*, genetic analysis of C. elegans mutants had already shown that, in the worm, physiological cell death required the activity of two "killer" genes, *ced-3* and *ced-4* (Ellis and Horvitz, 1986). The discovery that human *bcl-2* has functional and structural similarity to the anti–cell death gene *ced-9* demonstrated that programmed cell death in the nematode occurred by the same highly conserved mechanism as apoptosis in mammalian cells (Vaux et al., 1992; Hengartner and Horvitz, 1994). The killer gene *ced-3* was found to encode a cysteine protease with aspartic acid specificity (caspase) and was the archetype of a family of caspases that are the key effector proteins of apoptosis in mammalian cells (Yuan et al., 1993). The caspases are zymogens: they exist as inactive polypeptides that can be activated by removal of the regulatory prodomain and assembly into the active heteromeric protease. The key questions then became: What activates the caspases and what regulates their activation?

Genetic studies suggested that *ced-4* was required for *ced-3* function, whereas *ced-9* regulated apoptosis by preventing activation of the caspase encoded by *ced-3* (Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994). Recent biochemical data support these concepts, as CED-3 and CED-4 can physically interact (Chinnaiyan et al., 1997; Irmler et al., 1997), most likely by virtue of their N-terminal domains, which both contain a motif designated a caspase recruitment domain (CARD) (Hofmann et al., 1997). Furthermore, CED-9 and CED-4 have been found to directly interact when expressed in yeast and in mammalian cells (Chinnaiyan et al., 1997; James et al., 1997; Spector et al., 1997; Wu et al., 1997). Therefore, in C. elegans it appears that CED-4 is an adaptor protein that can receive an apoptotic signal, bind to pro-CED-3, and cause it to release its activated proteolytic domain. By binding to CED-4, CED-9 somehow prevents it from activating pro-CED-3.

### Regulation of Caspases by Adaptor Proteins and BCL-2 Family Proteins

While many mammalian homologs of CED-9 have been found (i.e., the BCL-2 family), and more than 10 mammalian caspases are known, until now homologs of CED-4 have been elusive. However, other mammalian caspase-activating adaptor proteins have been identified. For example, Mort-1/FADD interacts with certain caspases via shared death effector domains (DEDs) and RAIDD/CRADD interacts with other caspases via shared CARDs to transduce proapoptotic signals, such as those originating from TNF family receptors (Hofmann et al., 1997). In most cells, these cell death signals are poorly blocked by BCL-2, whereas BCL-2 is potent at inhibiting cell death triggered by other stimuli, such as withdrawal of growth factor, or up-regulation of p53 (Strasser et al., 1995). It may be that the ability of BCL-2 to inhibit apoptosis depends upon which adaptor molecule is involved in caspase activation.

Determining precisely how BCL-2-like proteins function has been surprisingly difficult. In various experimental systems, BCL-2 has been found to interact with calcineurin, p53 binding protein, lamin, NIP-1, -2, and -3, R-RAS, RAF-1, BAG-1, and galectin-3, as well as interacting with other BCL-2 family members. Recently it has been proposed that BCL-2 functions by forming pores to allow ions or small molecules to cross the outer mitochondrial membrane (Reed, 1997). As release of cytochrome c from the intermitochondrial space has been associated with apoptosis in a number of systems, and as this release is prevented by BCL-2, it has been hypothesized that the key function of BCL-2-like proteins is to somehow retain cytochrome c in the mitochondria (Kluck et al., 1997; Yang et al., 1997).
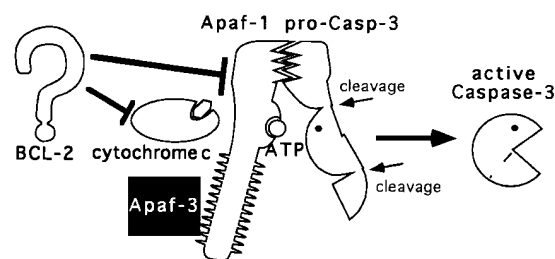


Figure 1. A Speculative Model for Caspase Activation

Activation of caspases such as caspase-3 occurs in the presence of dATP, cytochrome c (Apaf-2), Apaf-3, and Apaf-1. The caspase precursor is cleaved, allowing removal of the prodomain and assembly of other subunits into the proteolytically active conformation. Apaf-1 (or other adaptor molecules such as CED-4 in C. elegans) may interact with the caspase precursors via domains such as caspase recruitment domains (CARDs) or death effector domains (DEDs). The nature of Apaf-3 (depicted by black box) remains to be determined, as does the configuration of the physical interaction bewteen cytochrome c, Apaf-1, and Apaf-3. BCL-2-like proteins inhibit caspase activation by either binding directly to the adaptor protein (Apaf-1 or CED-4), preventing release of cytochrome c, or both.

The genetics of cell death in C. elegans and biochemical evidence of a direct interaction between CED-9 and CED-4 suggest that, before we can really understand how BCL-2-like proteins function, we will need to find the mammalian homolog(s) of CED-4. This search now appears to be over.

### A Human Protein Resembling CED-4

Using a biochemical approach to analyze cytosolic proteins that can activate caspase-3, Xiaodong Wang and coworkers have isolated a number of Apoptosis activating factors (Apafs 1–3) (Zou et al., 1997). Addition of dATP to these purified proteins results in cleavage and activation of the caspase-3 precursor. Earlier work revealed Apaf-2 to be cytochrome c, which was released from the mitochondria during homogenization. Apaf-3 is a 45 kDa protein that has not yet been cloned. Apaf-1 was found to resemble CED-4. It has an amino-terminal CARD domain and a 320 amino acid central portion with 22% identity and 48% similarity to CED-4. The carboxy-terminal half of Apaf-1 has 12 WD40 motifs that are believed to mediate protein–protein interactions.

Like CED-4, the peptide sequence of Apaf-1 contains regions that conform to the consensus for Walker A and B boxes, the nucleotide-binding p-loop motif. ATP may activate Apaf-1 by binding to this region. Supporting this notion, mutation of a residue predicted to be critical for nucleotide binding in the homologous region of CED-4 prevented it from promoting caspase activation in insect cells, as well as death of S. pombe overexpressing ced-4 (James et al., 1997; Seshagiri and Miller, 1997).

Using purified components, Zou et al. have shown that cytochrome c, dATP, the CED-4 homolog Apaf-1, and Apaf-3 are sufficient to activate pro-caspase-3, demonstrating that, in addition to CED-3 and CED-9, the CED-4 component of the apoptotic mechanism is highly conserved. Cytochrome c was found to associate with Apaf-1 in the presence or absence of dATP.

### Remaining Riddles

A model for caspase activation is shown in Figure 1. We still do not know precisely how BCL-2/CED-9-like proteins work. If they prevent apoptosis by blocking cytochrome c release, why would they need to bind to CED-4 proteins, and what is the role of BCL-2 in other locations, such as on the endoplasmic reticulum and nuclear membrane? On the other hand, if BCL-2-like proteins function by binding to and inhibiting CED-4, why would they need to prevent cytochrome c release?

It may be that BCL-2 proteins perform both functions. Another possibility is that BCL-2 may retain cytochrome c in the mitochondria indirectly. If cytochrome c was only one of a number of factors that can cause CED-4 to activate the caspases, induction of apoptosis by these alternate pathways might lead to a secondary loss of mitochondrial function, release of cytochrome c, and an amplification of the apoptotic cascade. In such a scenario, BCL-2-like proteins would only act as CED-4 inhibitors, with their ability to prevent cytochrome c release being indirect, by inhibiting the initial, premito-chondrial caspase activation.

Clearly, the experiments of Wang and coworkers have answered some questions but, like much good research, generated still more: Is dATP a regulator of apoptosis in physiological circumstances, or does another nucleotide play this role? What is the role of nucleotide binding—does it act as a switch to change the conformation and activity of Apaf-1, as occurs in G proteins, or is ATP used as energy to drive a reaction? What protein is responsible for cleaving the caspase precursor—is it autocatalytic, or can one of the Apafs act as a protease? What is the identity of Apaf-3—will it turn out to resemble known proteins, such as one of the proapoptotic BCL-2 family members, or will it be completely novel? What is the relationship, if any, between Apafs 1–3 and apoptosis-inducing factor (AIF), a 50 kDa caspase activating protein released from the mitochondria (Kroemer, 1997)? Do human BCL-2-like proteins bind to human CED-4-like proteins? Apoptosis remains one of the hottest areas of biological research. Chances are we won't have to wait long to find out.

### Selected Reading

Chinnaiyan, A.M., O'Rourke, K., Lane, B.R., and Dixit, V.M. (1997). Science 275, 1122–1126.

Ellis, H.M., and Horvitz, H.R. (1986). Cell 44, 817–829.

Hengartner, M.O., and Horvitz, H.R. (1994). Cell 76, 665–676.

Hofmann, K., Bucher, P., and Tschopp, J. (1997). Trends Biochem. Sci. 22, 155–156.

Irmler, M., Hofmann, K., Vaux, D., and Tschopp, J. (1997). FEBS Lett. 406, 189–190.

James, C., Gschmeissner, S., Fraser, A., and Evan, G.I. (1997). Curr. Biol. 7, 246–252.

Kluck, R.M., Bossywetzel, E., Green, D.R., and Newmeyer, D.D. (1997). Science 275, 1132–1136.

Kroemer, G. (1997). Immunol. Today 18, 44–51.

Reed, J.C. (1997). Nature 387, 773–776.

Seshagiri, S., and Miller, L.K. (1997). Curr. Biol. 7, 455–460.

Spector, M.S., Desnoyers, S., Hoeppner, D.J., and Hengartner, M.O. (1997). Nature 385, 653–656.

Strasser, A., Harris, A.W., Huang, D., Krammer, P.H., and Cory, S. (1995). EMBO J. 14, 6136–6147.

Vaux, D.L., Weissman, I.L., and Kim, S.K. (1992). Science 258, 1955–1957.

Wu, D.Y., Wallen, H.D., and Nunez, G. (1997). Science 275, 1126–1129.

Yang, J., Liu, X.S., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J.Y., Peng, T.I., Jones, D.P., and Wang, X.D. (1997). Science 275, 1129–1132.

Yuan, J.Y., Shaham, S., Ledoux, S., Ellis, H.M., and Horvitz, H.R. (1993). Cell 75, 641–652.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A., and Wang, X. (1997). Cell this issue, 90, 405–413.